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NEUROPROTECTIVE POTENTIAL OF ETHANOLIC ASHWAGANDHA ROOT EXTRACT, DOXYCYCLINE AND ELLAGIC ACID AGAINST ROTENONE INDUCED PARKINSON'S DISEASE: A *IN-VIVO* MODEL STUDY

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Keywords:

Parkinson's disease (PD), Behavioral alterations, Motor dysfunctions, ethanolic *Withania somnifera* root (EWSR) extract, Rotenone, Neurotoxicity, Neurotransmitters

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ABSTRACT: Parkinson's disease (PD) is the second most prevalent type of neurodegenerative disorder and is a chronic, progressive condition. In the current investigation, we used a rotenone (ROT)-developing rat model of PD to assess the neuroprotective efficacy of ethanolic *Withania somnifera* root extract (EWSR) in comparison to the known neuroprotectants *i.e.*, Doxycycline (Doxy), and ellagic acid (EA) individually and in combination (EWSR-Doxy-EA). Male Wistar rats were given ROT at a dose of 2.5 mg/kg body weight daily for 4 weeks to develop a PD *in-vivo* model. Once the PD model is confirmed, the rats were treated with individual neuroprotectants for next 5 weeks and kept under observation for next 7 days for reversal of PD. ROT administration has altered behavioural parameters (Cognitive, motor dysfunction, sniffing, grooming, raring, crossing, *etc*.) and neurotransmitter profile (Acetylcholine, epinephrine, nor-epinephrine, dopamine, glutamate and aspartate) in rats. The administration of neuroprotectants has ameliorated the deleterious effects of rotenone and restored cognitive and motor control by rectifying the levels of neurotransmitters which are responsible for the functions. EWSR protective efficacy was significantly better to that of the known neuroprotectants (Doxy and EA) and EWSR efficacy increased significantly when combined with the other neuroprotectants. Based on the study observations, it can be inferred that EWSR is a good neuroprotective agent and can act as a potential alternative therapeutic approach individually or in combination with known drugs in treating PD and other neurodegenerative diseases.

INTRODUCTION: Parkinson's disease (PD) is a progressive, late-onset neurodegenerative condition marked by the development of fibrillar cytoplasmic inclusions containing α-synuclein and ubiquitin and relatively selective nigrostriatal dopaminergic loss.

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Alpha-synuclein (aSyn) misfolding and aggregation are hallmarks of the neurodegenerative illnesses Parkinson's disease (PD) and dementia with Lewy bodies (DLB) [1-4].

Although the exact cause of PD is unknown, it is thought to result from the interplay of hereditary and environmental factors [1-3]. Epidemiological research indicates that PD risk may be increased by exposure to environmental factors like pesticides [5-8]. PD and mitochondrial dysfunction have both been connected [2-5].

In PD brain, muscle, and platelets, there are systemic decreases in the activity of complex I of the mitochondrial electron transfer chain (ETC) [2-5]. The discovery that MPP+ (1-methyl-4-phenyl-2,3-dihydropyridines), the active metabolite of the parkinsonism toxin N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), serves as a complex I inhibitor provides additional evidence for mitochondrial dysfunction in PD [9, 10].

Rotenone is a naturally occurring compound obtained from the roots, seeds, and stems of several tropical plants, such as the Derris, Tephrosia, Lonchocarpus, and Mundulea species [10-13]. Rotenone was found in South America and Southeast Asia hundreds of years ago, and it is currently an active component in thousands of insecticides and piscicides. Since 2000, when Betarbet *et al*.[12] replicated the main symptoms of PD in rotenone-infused rats, rotenone has received a lot of attention. Since then, rotenone has drawn much interest because it is one of the environmental neurotoxins linked to the etiopathogenesis of PD [14-18].

Tetracyclic antibiotic Doxycycline exhibits neuroprotective effects that were once thought to be related to its anti-inflammatory characteristics and prevention of amyloid aggregation, reducing the formation of reactive oxygen species originating from mitochondria and prevents the aggregation and seeding of recombinant alpha-synuclein (aSyn), a new mechanism by which it may exert its neuroprotective properties has been postulated [19, 20]. Finally, it is reported that doxycycline triggers a neuroprotective mechanism by restoration of dopaminergic function in a rat model of Parkinson's disease. There are few compelling evidences that doxycycline therapy may be a successful method for treating synucleinopathies [18-22].

In fruits and nuts such as grapes, strawberries, raspberries, pomegranates, and walnuts, ellagic acid (EA) is a polyphenol and a naturally occurring dimeric derivative of gallic acid, can be discovered [23-27]. This polyphenol functions as an anti-inflammatory and antioxidant in mammalian cells [25, 26]. *In-vitro* studies have shown EA to have anticholinesterase and antioxidant properties. EA's neuroprotective function has been documented in a variety of experimental models [24]. Additionally, EA can lessen cognitive disruption after scopolamine injection, prevent cognitive deficits brought on by traumatic brain damage, and restore memory loss in a 6-hydroxydopamine-induced PD model. Neuroprotective potential of EA is by improving cholinergic transmission in an intrahippocampal Aβ-25-35-injected rat model of AD [22-28].

The herb *Withania somnifera* (WS), which is a member of the Solanaceae family, is popularly known as Ashwagandha or Indian ginseng. It's been part of Ayurveda or herbal medicine in South Asia, like India, Pakistan, and Afghanistan, over centuries, and now it's cultivated in South Africa and other nations [29-30]. WS has a wide range of medicinal uses, including anti-diabeitc, anti-inflammatory, antioxidative, anti-stress and memory-improving properties (nootropic) with beneficial effects on the immunological system, circulatory system, central nervous system, *etc*. [31-37] The root extract of WS contains significant amounts of withaferin A, withanolides, withanone, and other flavonoids as well as other compounds with strong antioxidant properties [33-35]. WS root extract boosts mental recall capacity after memory loss brought by foot shock stress, diabetes, amyloid, and scopolamine [30, 33-37]. This study's primary objective is to evaluate the beneficial effects of EWRE compared to known neuroprotective agents *i.e.*, doxycycline and ellagic acid against rotenone-induced neurodegeneration.

MATERIALS AND METHODS:

Materials: Wistar rats, Rotenone (Rot, ([2R-(2',6',12')]) -1,2,12,12a-tetrahydro -8,9-dimethoxy-2-(1-methylethenyl) -[1]benzopyran[3,4-b] furo [2,3-h] [1] benzopyran-6(6aH)-one)), Doxycycline (Doxy), Ellagic acid (EA),*Withaniasomnifera*(WS) root powder, Ethanol, Folin-Ciocalteu solution, Alkaline copper sulphate, Bovine serum albumin (BSA), Ninhydrin, Guanidinecarbonate, Lead acetate, Sodium hydroxide, double distilled water (ddH_2O), Hydrochloric acid, 2,4 dinitrophenyl hydrazine, Stannous chloride (SnCl2), Isobutanol, Citrate buffer.

Methodologies:

Animal Experimentation: Fifty-four male Wistar rats weighing 160–200 grams were procured from the National Institute of Nutrition (NIN),

Hyderabad, India. The rats were kept in quarantine before housing them in individual polypropylene cages and were fed with a standard pellet diet (NIN, Hyderabad) and water supplied *ad libitum* throughout the experiment. We got the ethical committee approval for the proposed study (IAEC: CPCSEA/IAEC/JLS/17/03/22/01 2). The rats were divided into four groups with six animals per group. The first group (controls) received sunflower oil, the second to sixth group were administered daily with rotenone (Rot) dissolved in sunflower oil (2mg/kg bw) through intraperitoneal route for 4 weeks and the groups from seventh to ninth were also treated individually with EWSR (100mg/kg bw), Doxycycline (40mg/kg bw) and Ellagic acid (40mg/kg bw) for 5-6 weeks through gavage. The rats were maintained for 70 days right from day 1 to day 28 of neurotoxin-induced and later up on confirmation of Parkinson's disease symptoms, the neuroprotectants were administered for next 5 weeks and 1 week under observation post neuroprotective treatments. The rat pups were sacrificed by cervical dislocation, and their brains were dissected out and immediately transferred to ice-cold conditions (initially at 4°C and then to −20°C) for further studies. The behavioural studies were done on day 30 and day 70 of the experiment.

Behavioural and Locomotor Activity Studies:

Rotarod: The rotarod test, which measures performance, compels the animal to move because of the rotating rod. The rotarod performance test is used to measure riding duration (in seconds) or motor coordination endurance and to evaluate the balance and coordination activity of the relevant pharmaceutical impact on animals. The experimental animals were mounted on a cylindrical rod that rotated horizontally; normally, the creatures prefer to remain stationary in order to avoid falling to the ground. The amount of time an animal spends on the rotating rod demonstrates its balance, coordination, health, and motor activity. The rotarod is kept in working order by a motor with a variable speed [39-41].

Maze Learning: Animal learning and adaptability to environments and difficulties were studied using the Hampton Court maze as a research instrument. The experimental animals were given free license to navigate the maze's many-branched passages in search of a way to get reinforcements. It is the primary method used to study spatial learning and a common experiment in behavioural labs. Rats from all experimental groups (Control, Rot, Rot-protectants) were permitted to spend time in maze training given before the recording the time to accomplish the goal. The rats were deprived for 12 hours before to the maze learning experiment (*i.e.*, food) [39-41].

Open Reading Test: The open field test assessed various behavioural reactions, including anxiety, emotional reactivity, and locomotor activity. This test was performed on rats before and after rotenone therapy, with or without neuroprotectants. Each rat was kept in an open area that measured 50 by 50 by 60 cm, with a floor divided into squares by black lines that were each 12 by 12 cm in size. The number of squares crossed by each rat with all four paws, rearings (when the rodents stood on their hind legs), wall raising, groomings (face washing, forepaw licking, and head stroking), sniffing, and wall sniffing were all counted every five minutes for fifteen minutes. Each rat's square crossings and rearings were counted as indicators of locomotor activity. Still, the number of groomings and sniffings was considered an indicator of behavior, or emotionality. After examining each animal, the open field was cleaned with a damp cloth [40-41].

Protein Estimation: Modified Lowry's technique (1951) was used to estimate the amount of protein. Using bovine serum albumin (BSA; 1 mg/ml) as the standard protein, a standard protein graph was created. From 0.05 to 1 mg/mL, BSA workable solutions were made. Alkaline copper sulphate reagent (analytical reagent) was added to a predetermined volume of 0.2 ml of diluted protein solution from several test tubes, and the mixture was thoroughly mixed. The mixture was next allowed to sit at room temperature for 10 minutes. The aforementioned mixture received 0.2 ml of reagent Folin-Ciocalteu solution (reagent solutions), which was then incubated for 30 minutes. At 660 nm, the optical densities (ODs) of solutions containing known and unidentified proteins were measured [38-40].

Quantification of Neurotransmitters:

Quantification of Acetylcholine: The brain tissues were homogenised in 3% perchloric acid and

centrifuged for 10 min. at 1000 rpm. The modified approach developed by Long *et al*. was used to examine the levels of Ach [38-40].

Quantification of Catecholamines: The brain tissue homogenates were produced using a 0.25 M sucrose solution and centrifuged for 10 min. at 1000 rpm. Dopamine (DA, excitation: 310 nm; emission: 410 nm), nor-epinephrine (NE, excitation: 387 nm; emission: 487 nm), epinephrine (EPN, excitation: 410 nm and emission: 500 nm) levels were measured in the hippocampus and midbrain of rats exposed to Rotenone [38-40].

Quantification of Excitatory Neuro-transmitters (Glutamate and Aspartate):

Glutamate: The modified methods of Ciarlose 19, 38 and Margret et al. 43 were applied to measure glutamate. After being homogenized in 3% perchloric acid, brains were centrifuged at 1000 rpm for ten minutes at 4 °C. The supernatant was removed, mixed with 1% ninhydrin, and heated for ten minutes before being added to the mixture along with 0.4 ml of guanidine carbonate, 1 ml of 100 mM lead acetate, 0.5 ml of 1 N NaOH and six milliliters of dH_2O. After adding 0.1% of 2,4 dinitrophenyl hydrazine dissolved in 0.01N HCl to the mixture, it was allowed to react for 30 minutes at ice-cold temperatures. The colour intensity at 420 nanometers was measured using an ultraviolet-visible spectrophotometer (ThermoFisher) [38-40].

Aspartate: Brains were homogenized in 1 ml of 3% perchloric acid and centrifuged at 1000 rpm for ten minutes. The supernatant was then transferred to a new tube, to which 2 ml of citrate buffer, 1 ml of 1% ninhydrin solution, and 1 ml of 1% SnCl2 were added. The mixture was then vortexed, heated for fifteen minutes at 100°C, and then cooled on Isobutyl alcohol was afterward added, and 6 ml was vortexed. At 570 nm, the alcohol layer was collected, and OD was noted [38-40]. The findings were represented as g of monoamine/g wet tissue weight.

RESULTS AND DISCUSSION: To understand the pathogenic foundation of PD, rotenone, MPTP and 6-OHDA have all been extensively studied in animal models. Rotenone, a well-known mitochondrial complex I inhibitor, is frequently employed to investigate PD's pathophysiology and assess the efficacy of prospective innovative treatments for condition [1-5]. Rotenone is a highly lipophilic PD mimic that can be used to study lewy-body development because, unlike other PD mimics such 1-methyl, 4-phenyl, 1, 2, 3, 6-tetrahydropyridine (MPTP), it does not need to cross the blood-brain barrier [9]. In addition, it has been shown to accelerate oxidative stress, neuroinflammation, alpha-synuclein aggregation, impaired autophagy, and behavioural deficits [10-15].

Withania sominfera (L.) (WS) plant has been extensively studied over the years due to their broad spectrum therapeutic potential and few side effects compared to synthetic compounds, herbal extracts with several active principles have become favoured prophylactics [43-47]. To treat convulsive disorders, tremors, and pathophysiological signs resembling the contemporary concept of neurodegenerative illnesses, *Withania somnifera* root extract (WSRE) has been utilised in traditional Indian medicine [43-47]. There is ample evidence that WS plays a significant part in PD. It is one of the Ayurvedic plants extensively explored for PD in both *in-vitro* and *in-vivo* settings [45-47].

It has been demonstrated that a standardized extract of WS can significantly lessen the locomotor defects and lethality of rotenone-induced Parkinsonism in *Drosophila melanogaster* by reducing oxidative stress, enhancing cholinergic function, and improving mitochondrial respiratory mechanisms [37].

WS also dramatically reduced rotenone-induced parkinsonism in the brain through its anti-inflammatory and anti-oxidant properties and by resolving mitochondrial dysfunctions in the striatum and cerebellum regions. The striatum's dopamine levels and neurotransmitter functioning have both been restored due to all these modifications.

In the past, it was discovered that WS has unique anti-oxidant qualities that helped reverse PD, increase striatal catecholamine levels, and reverse functional deficits. A further prediction made by the study is that the number of D2 dopaminergic receptors in the striatum may increase [29, 30]. These receptors are known to have a compensatory function following Parkinsonism induction [34-36, 45].

Additionally, a human PD model has demonstrated that Ashwagandha dramatically and dose-dependently reverses neurodegenerative symptoms [44]. Additionally, *Withania somnifera* root extract (WSRE) has shown to increase catecholamine content in MPTP-induced Parkinsonism in mouse brain. Its leaf extract has been demonstrated to reduce oxidative damage and modulate physiological abnormalities in a mouse model of PD [43-47].

Prakash *et al* (2014) [48] has demonstrated the ability of WSRE in the restoration of dopamine levels in *Substantia nigra* and motor stability in a Maneb-paraquat-induced PD mouse model by reducing the neuroinflammation, oxidative damage, and apoptosis. Furthermore, WSRE increased tyrosine hydroxylase activity, shielded dopaminergic neurons, simultaneously reduced inducible nitric oxide synthase (iNOS) enzyme activity, and decreased apoptotic stimuli upon WS's administration in Mouse PD model [45-48].

Investigations into the active components and metabolites of WS and other natural products are ongoing in an effort to identify any substances that may improve the therapeutic effects of experimental Parkinsonism by assisting in the synthesis, release, and uptake of dopamine.

Behavioural Studies: Rotarod

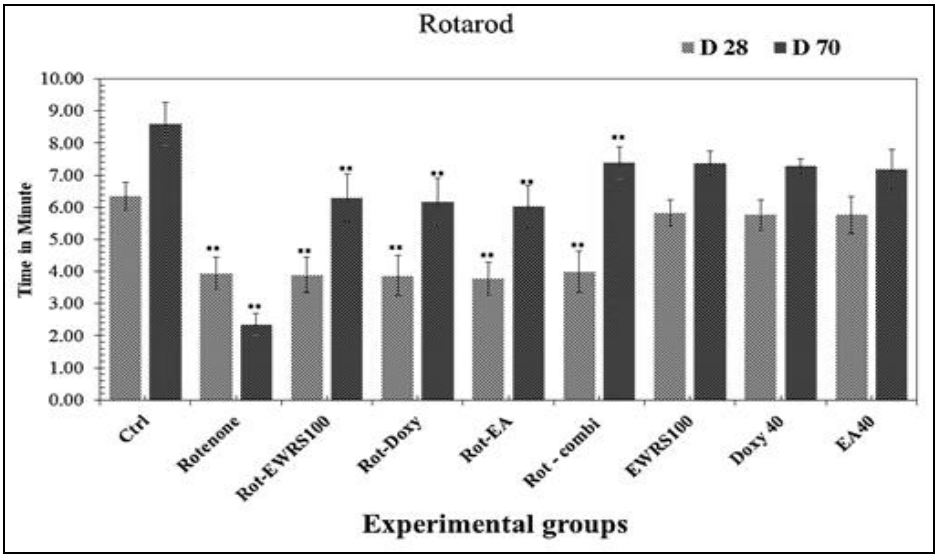

FIG. 1: ROTENONE EFFECTS ON THE MOTOR ACTIVITY IN RATS BEFORE AND AFTER ADMINISTRATION OF NEUROPROTECTANTS. The values are mean ± standard deviation of 6 (n = 6) individual observations; one-way ANOVA, **P <0.01 indicates significant observations.

When rats were subjected to repeated exposure to rotenone, it caused worsening effects. When the rats were used in a rotarod trial, it revealed that they had trouble balancing and walking on the rotatory rob at a constant speed.

Compared to controls and rats getting EWSR, Doxy, EA, and EWSR-Doxy-EA, rats receiving rotenone could not balance for longer. EWSR and other neuroprotectants improved balancing and motor learning ability in the treated rats. EWSR treatment, both alone and in combination, reduced the rotenone-induced neuromotor deficits.

Maze Learning: Chronic exposure to rotenone-induced deteriorating effects in rats, and when put in a maze learning experiment, it showed that they were facing difficulty in navigating themselves through the maze and toward reaching the target (in this case, its food pellets).

Rotenone-administered rats took longer than the controls and rats receiving EWSR, Doxy, EA and EWSR-Doxy-EA. The rats treated with EWSR and other neuroprotectants showed improved cognition and maze learning ability.

The rotenone-induced neuromotor and cognitive alterations were ameliorated on EWSR administration, *i.e.*, alone and in combination.

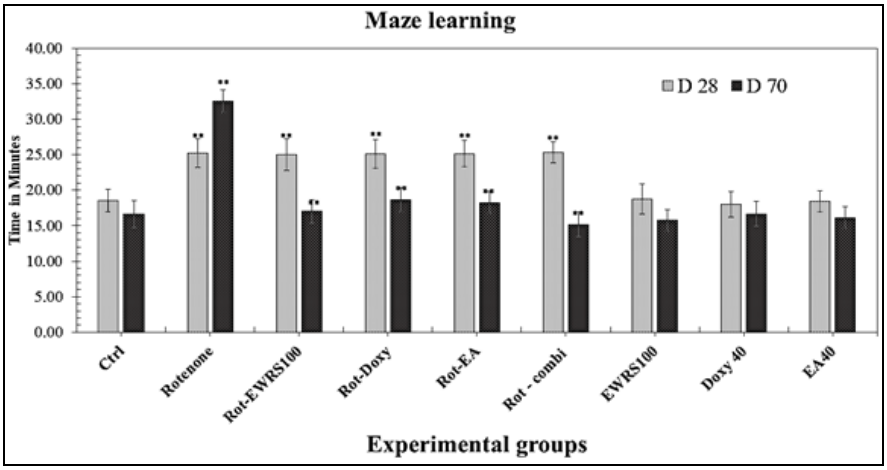

FIG. 2: ROTENONE EFFECTS ON THE COGNITIVE FUNCTIONS IN RATS BEFORE AND AFTER ADMINISTRATION OF NEUROPROTECTANTS. The values are mean ± standard deviation of 6 (n = 6) individual observations; one-way ANOVA, **P <0.01 indicates that the observations made are significant.

Open Field Test: The behavioural studies gave insights into an animal's stress and anxiety. By the open-field test, we made an observation that the rotenone-induced rats showed lethargy, and motor discomfort in crossing, grooming, rearing, and sniffing when compared to the control and the rats receiving EWSR, Doxy, EA and neuroprotectant combination (EWSR-Doxy-EA). The crossing, grooming, rearing, and sniffing patterns increased in rats treated with EWSR and the patterns were very much similar to that of the controls and other known neuroprotectants (Doxy, EA); based on the results, it is clear that the EWSR and neuroprotectant combination (EWSR-Doxy-EA) works as a neuroprotectants against rotenone-induced toxicity. The motor activity in the rats treated with rotenone gradually decreased compared to that of controls and rats receiving EWSR, Doxy, EA, and neuroprotectant combination (EWSR-Doxy-EA). The rats' receiving the EWSR alone and in combination showed synchrony and bettered motor and cognitive activities.

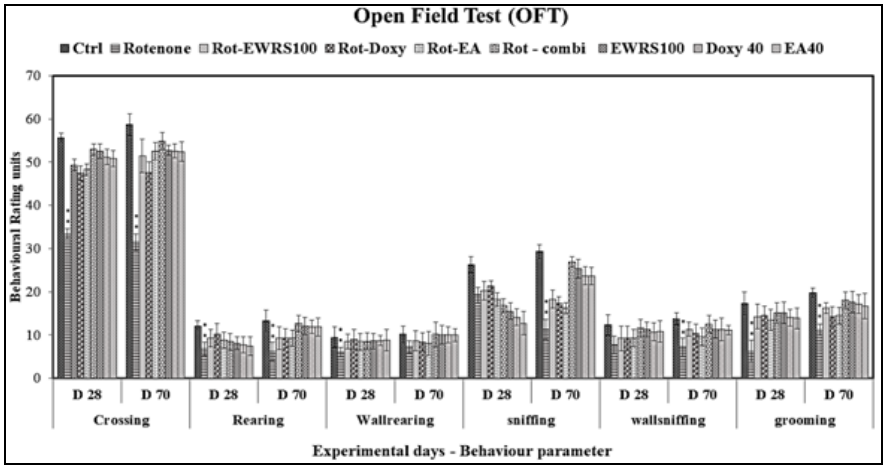

FIG. 3: ROTENONE-INDUCED BEHAVIOURAL ALTERNATIONS IN RATS BEFORE AND AFTER ADMINISTRATION OF NEUROPROTECTANTS. The values are mean ± standard deviation of 6 (n = 6) individual observations; one-way ANOVA, **P <0.01 indicates significant observations.

Acetylcholine: The major neurotransmitter in motor neurons is acetylcholine (Ach), and this Ach aids in controlling the activity in neuromuscular junctions; hence the levels of Ach must be in higher to maintain proper motor functions. However, there was gradual loss of motor control in the rats as the levels of Ach decreased in both hippocampus and midbrain regions of rats on rotenone administration. Treatment with EWSR and other neuroprotectants improved motor activity and Ach levels in rats exposed to rotenone. Although the activity and Ach levels in the groups

receiving the EWSR and other neuroprotectants were less compared to the controls and groups receiving only neuroprotectants, its treatment was still able to restrict the depletion of Ach and loss of motor control. The effect of rotenone was observed in both the hippocampus and midbrain regions, and as a result, the Ach levels dropped drastically, causing motor dysfunction and behavioural deficits.

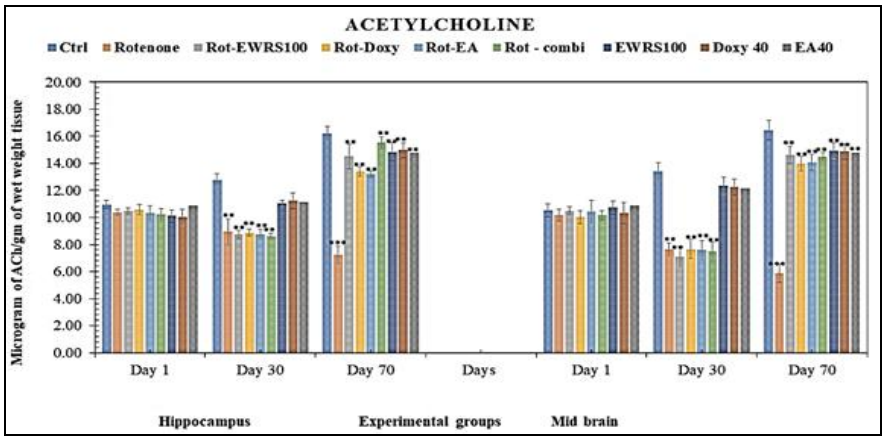

FIG. 4: ROTENONE INDUCED COGNITION AND MOTOR DYSFUNCTIONS BY ALTERING LEVELS OF ACETYLCHOLINE IN DIFFERENT REGIONS OF RAT BRAIN BEFORE (DAY 1- BEFORE ADMINISTRATION OF ROTENONE; DAY 30 - AFTER ROTENONE ADMINISTRATION AND BEFORE ADMINISTRATION OF NEUROPROTECTANTS) AND AFTER ADMINISTRATION OF NEUROPROTECTANTS (DAY 70 – POST NEUROPROTECTANT ADMINISTRATION). The values are mean ± standard deviation of 6 (n = 6) individual observations; one-way ANOVA, **P <0.01; ***P< 0.001 indicates that the observations made are significant and highly significant.

Catecholamines

Dopamine: On continuous rotenone exposure, the levels of DA in the brain's midbrain and hippocampus steadily dropped. Rotenone-treated rats displayed considerably lower DA levels than the controls, EWSR, Doxy, and EA-treated rats. With dose and duration, the EWSR, Doxy, EA, and therapy all dramatically raised the levels of DA. The above chart shows how effective EWSR is at protecting against damage because it can restore the levels close to the levels seen in the control and individual neuroprotective groups. In contrast to the controls and the rats who received EWSR, Doxy, EA, and EWSR-Doxy-EA, the rats exposed to rotenone for an extended period showed a significant decrease in 5HT levels in both the midbrain and hippocampal areas of the brain.

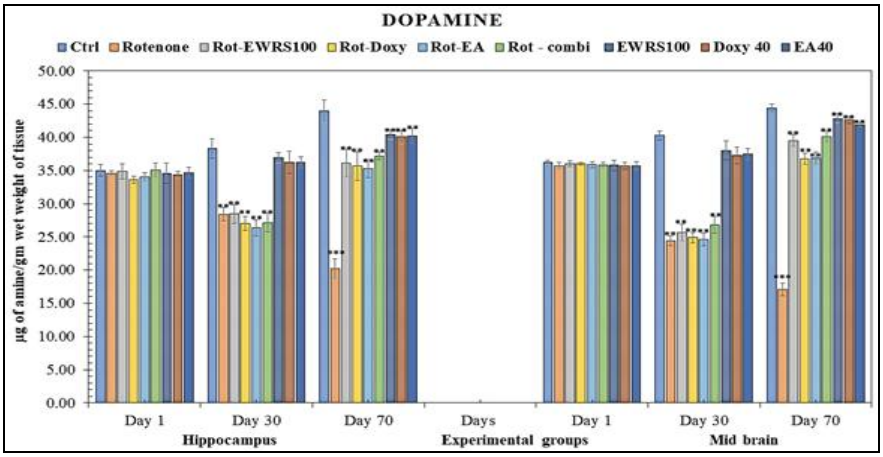

FIG. 5: ROTENONE INDUCED NEUROINFLAMMATION AND MOTOR DYSFUNCTION BY ALTERING THE LEVELS OF DOPAMINE IN DIFFERENT REGIONS OF RAT BRAIN BEFORE (DAY 1- BEFORE ADMINISTRATION OF ROTENONE; DAY 30 - AFTER ROTENONE ADMINISTRATION AND BEFORE ADMINISTRATION OF NEUROPROTECTANTS) AND AFTER ADMINISTRATION OF NEUROPROTECTANTS (DAY 70 – POST NEUROPROTECTANT ADMINISTRATION). The values are mean ± standard deviation of 6 (n = 6) individual observations; one-way ANOVA, **P <0.01; ***P< 0.001 indicates that the observations made are significant and highly significant.

Nor-epinephrine: Rotenone exposure over an extended period of time has decreased the levels of NE in the hippocampus and midbrain. When compared to the levels in the rats that received EWSR, Doxy, EA, and Combi, the levels were much lower. With dose and duration, the EWSR therapy markedly raised NE levels. The rise in NE levels is a sign that EWSR is protecting rats' developing brains effectively and can manage neuroinflammation and the dysfunctions it causes. The EWSR-treated rats demonstrated that their levels were comparable to those of the control, Doxy, EA, and Combi groups.

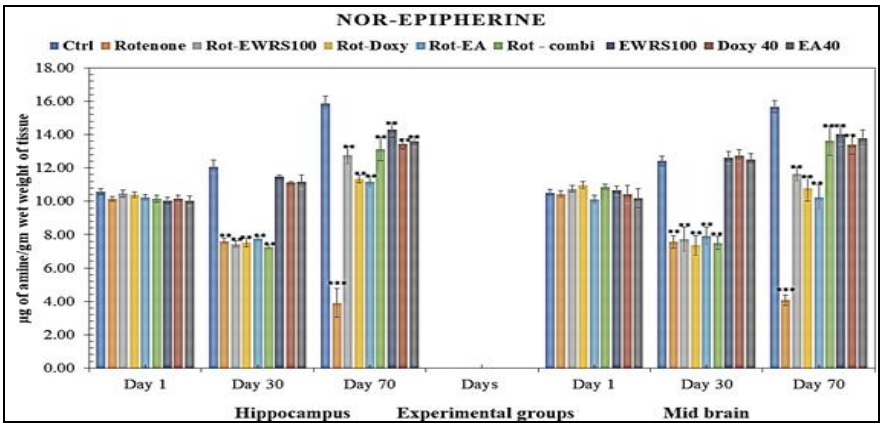

FIG. 6: ROTENONE-INDUCED NEUROINFLAMMATION AND ALTERATION IN NOR-EPINEPHRINE LEVELS IN DIFFERENT REGIONS OF RAT BRAIN BEFORE (DAY 1- BEFORE ADMINISTRATION OF ROTENONE; DAY 30 - AFTER ROTENONE ADMINISTRATION AND BEFORE ADMINISTRATION OF NEUROPROTECTANTS) AND AFTER ADMINISTRATION OF NEUROPROTECTANTS (DAY 70 – POST NEUROPROTECTANT ADMINISTRATION). The values are mean ± standard deviation of 6 (n = 6) individual observations; one-way ANOVA, **P <0.01; ***P< 0.001 indicates that the observations made are significant and highly significant.

Epinephrine: Chronic rotenone administration steadily elevated EPN levels, which were considerably higher in the cerebral cortex and hippocampus than in the controls and rats who received EWSR. With dose and time, the EWSR therapy greatly reduced the levels of EPN. The levels of EPN in the rats given EWSR, Doxy, EA, and Combi were nearly identical to control and individual neuroprotectant groups, demonstrating the effectiveness of this protective agent. In the midbrain and hippocampus, rotenone significantly impacts aspartate buildup and receptor stimulation; the reversal effect of EWSR on EPN levels is comparable to that of the well-known neuroprotectants Doxy and EA.

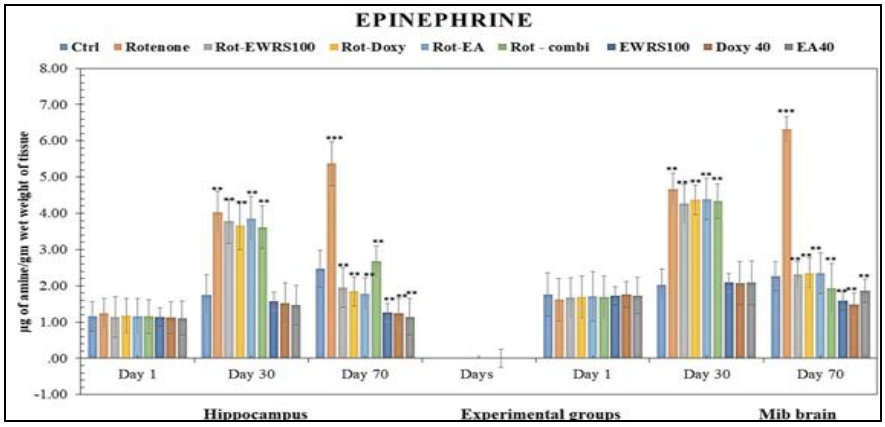

FIG. 7: ROTENONE-INDUCED NEUROINFLAMMATION AND ALTERATION IN EPINEPHRINE LEVELS IN DIFFERENT REGIONS OF RAT BRAIN BEFORE (DAY 1- BEFORE ADMINISTRATION OF ROTENONE; DAY 30 - AFTER ROTENONE ADMINISTRATION AND BEFORE ADMINISTRATION OF NEUROPROTECTANTS) AND AFTER ADMINISTRATION OF NEUROPROTECTANTS (DAY 70 – POST NEUROPROTECTANT ADMINISTRATION). The values are mean ± standard deviation of 6 (n = 6) individual observations; one-way ANOVA, **P <0.01; ***P< 0.001 indicates that the observations made are significant and highly significant.

Glutamate: An excitatory neurotransmitter that is most prevalent in the brain is glutamate. Compared to the controls and the rats receiving EWSR, the rotenone-treated rats have significantly higher amounts of glutamate in both their brains' midbrain and hippocampal regions. Because EWSR effectively lowers glutamate levels over time and with dose, the extract can be regarded as an anti-excitatory source. The midbrain and the hippocampus are highly affected by rotenone's effect on glutamate buildup. EWSR's ability to reverse this effect is comparable to the well-known neuroprotectants Doxy and EA.

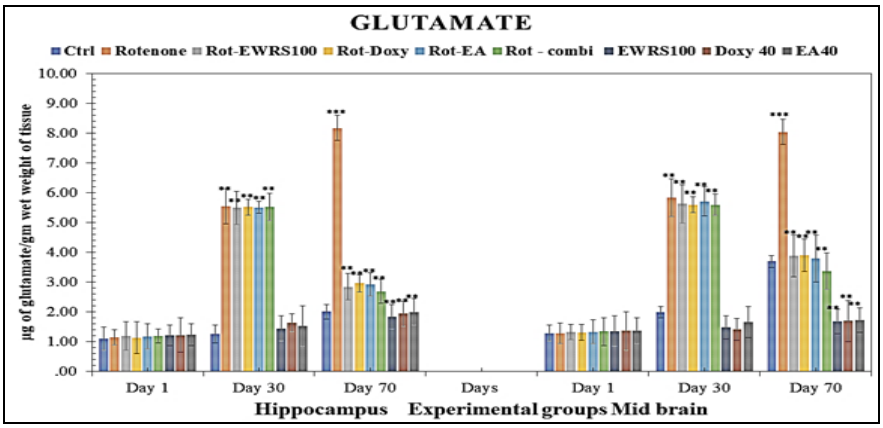

FIG. 8: ROTENONE-INDUCED NEUROINFLAMMATION AND ALTERATION IN GLUTAMATE LEVEL IN DIFFERENT REGIONS OF RAT BRAIN BEFORE (DAY 1- BEFORE ADMINISTRATION OF ROTENONE; DAY 30 - AFTER ROTENONE ADMINISTRATION AND BEFORE ADMINISTRATION OF NEUROPROTECTANTS) AND AFTER ADMINISTRATION OF NEUROPROTECTANTS (DAY 70 – POST NEUROPROTECTANT ADMINISTRATION). The values are mean ± standard deviation of 6 (n = 6) individual observations; one-way ANOVA, **P <0.01; ***P< 0.001 indicates that the observations made are significant and highly significant.

Aspartate: Another excitatory neurotransmitter in the brain is aspartate, and rats exposed to rotenone had steadily higher aspartate levels. The levels in the brain's cerebral cortex and hippocampus of the controls and the rats who received the EWSR were noticeably higher. Aspartate levels were successfully reduced by the EWSR treatment with dose and duration administered concurrently. Animals receiving EWSR had aspartate levels between the control and rotenone groups. Both the midbrain and the hippocampus showed a significant effect of rotenone on the buildup of excitatory neurotransmitters like aspartate; the reversal effect of EWSR on aspartate levels is comparable to those of the well-known neuroprotectants, namely Doxy and EA.

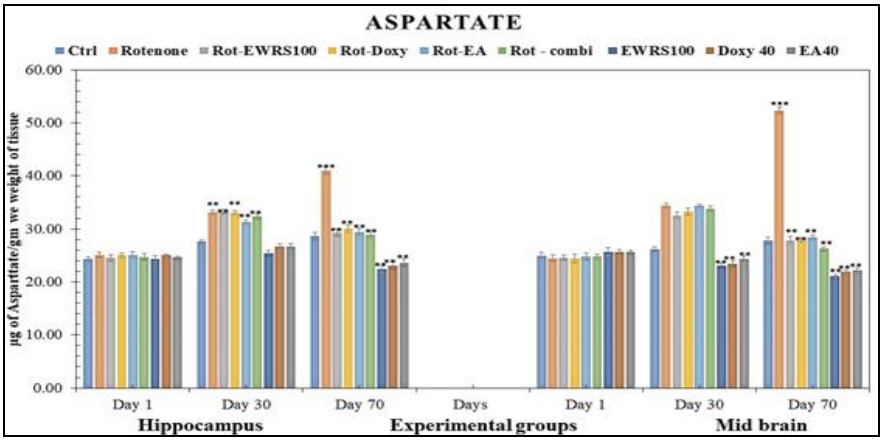

FIG. 9: ROTENONE-INDUCED NEUROINFLAMMATION AND ALTERATION IN ASPARTATE LEVEL IN DIFFERENT REGIONS OF RAT BRAIN BEFORE (DAY 1- BEFORE ADMINISTRATION OF ROTENONE; DAY 30 - AFTER ROTENONE ADMINISTRATION AND BEFORE ADMINISTRATION OF NEUROPROTECTANTS) AND AFTER ADMINISTRATION OF NEUROPROTECTANTS (DAY 70 – POST NEUROPROTECTANT ADMINISTRATION). The values are mean ± standard deviation of 6 (n = 6) individual observations; one-way ANOVA, **P <0.01; ***P< 0.001 indicates that the observations made are significant and highly significant.

Earlier *in-vitro* studies (unpublished or under publication) showed the anti-inflammatory, cytoprotective properties of EWSR against rotenone induced deterimental effects. The present study was designed to develop *in-vivo* model of parkinsons disease and evaluate the neuroprotective properties of EWSR, Doxy, EA alone and in combination against the rotenone induced neurodegenerative effects such as behavioural, cognitive, and neurotransmitters profile. When comparing the brain lysates of the Rotenone group to the control and protectant groups, a decreased concentration of total proteins was seen. The findings of our study are consistent with the body of literature that documents changes in protein levels in rotenone-induced brains' hippocampus and midbrain. One of mammals' most fundamental learning and cognition types is nonassociative behavioural habituation.

In experimental models, Banala *et al*. demonstrated the use of the open field test as a model for the concurrent assessment of anxiety and memory (rodents). Compared to subsequent exposures, the rats given an open field test exhibit greater geographic exploration for information collecting and cognition [40]. A new recognition process is also created when exposed to a new environment. This process involves greater awareness and comparing the novel spatial information with previously examined locations stored in the brain. As a result, the delayed reaction to subsequent exposures is considered a measure of memory of habituation. Hippocampal dysfunction may be linked to learning when decision alternatives and their results are associated.38-40

The rotarod test measures an animal's ability to maintain motor coordination over an extended period; several elements, including visual perception, cutaneous sensation, brain activity, and neuromuscular integration, are necessary to elicit this behaviour. The current study reports decreased motor coordination activity **Fig. 1** in experimental pregnant rats treated with rotenone at 2 mg/kg bw for 28 days (rotenone group). Motor coordination analyses were performed on a rotarod apparatus, which is consistent with earlier reports showing decreased motor activity and coordination in male Wistar rats treated with rotenone. Chronic Rotenone exposure caused motor impairment in the rats, although controls such as EWSR Doxy, EA, and Combi given to rats after Rotenone therapy demonstrated normal motor coordination when placed on the rotarod apparatus. The differences seen in the rotenone-treated rats were limited to how EWSR and other neuroprotectants were administered.

In contrast to the normal behaviour patterns seen in the control and neuroprotectant-treated groups, rotenone administration changed motor coordination activity, emotional response, and goal-oriented maze learning. The maze was created in **Fig. 2** so that researchers could study how well rodents could learn and retain food locations. According to various research groups, the learning task is carefully selected to learn a lot about the neurochemical, neuroanatomical, and neurophysiological underpinnings of behavioural association [16, 17, 26].

The current investigation results indicated that rotenone exposure harmed maze performance that was goal-oriented [40-43, 45]. Although the rotenone-treated rats could complete the task, they did so much more slowly than the control and neuprotectant-treated rats due to cognitive and memory deficits [45]. The behavioural assessment used in our investigation, which was based on a maze learning task, was comparable to that used in earlier studies. The rats exposed to rotenone had different maze learning abilities in relation to reference and working memory tasks, which was remedied by concurrent EWSR administration.

A more compelling and significant amelioration of fluoride toxicity on short and long-term memory in a dosage-dependent manner is shown by the decrease in delay in the discovery of the food on reference and working memory trials. The open field **Fig. 3** test has been used frequently to measure rodents' spatial memory and anxiety. Using motor activity characteristics, such as delay, grooming, and rearing, the emotional response in the open field test was evaluated [38, 40]. In addition to modified behaviours like scratching and smelling, motivated behaviour in rats includes observation of important body motions and positions, including sitting, standing, and walking. Failure of experimental rats to become accustomed to a task in an open field after prenatal and

lactational exposure to rotenone caused cognitive damage. We concluded that these rats exhibited an intact recollection of habituation based on the crossing, rearing, and sniffing behaviours displayed by the control and neuroprotectant receiving groups. Since the hippocampus is responsible for processing memory and habit in the open field test, exposure to rotenone results in hippocampal cell death due to excitotoxicity and neurooxidative stress [38, 40, 41].

Acetylcholine (Ach) levels in rotenone-induced rats were discovered to be abnormally low, as opposed to normal levels in rats that received controls or specific neuroprotectants. The rats showed dopamine levels that were significantly higher than those of the rotenone group and very similar to controls after taking EWSR and other neuroprotectants. The reduced Ach levels may affect the rat's motor coordination, open-field upbringing, and scratching tendencies. According to the literature evaluation, complex stereotype behaviours in rats are unquestionably related to Ach levels and persistent rotenone poisoning **Fig.4** [38, 40, 41].

Dopamine levels were found to be abnormally low in rotenone-induced rats, in contrast to normal levels in rats that received controls or individual neuroprotectants. After receiving EWSR and other neuroprotectants, the rats displayed dopamine levels that were considerably greater than those of the rotenone group and close to those of controls. The rat's motor coordination, open-field rearing, and scratching habits may all suffer as a result of the lower DA levels. Dopamine and persistent rotenone poisoning are definitely linked to complex stereotype behaviours in rats, according to the literature review **Fig. 5** [41].

Motor control, depression, memory, and cognition are all maintained by neurotransmitters like EPN and NE. The current investigation shows that chronic rotenone therapy dramatically changed the levels of EPN and NE in rats, increasing EPN levels and decreasing NE levels, respectively. In contrast, neuroprotectant-administered animals and controls had lower EPN levels and higher NE levels, indicating that rotenone-induced changes in neurotransmitter levels can be limited by the EWSR and other neuroprotectants **Fig. 6** and **7.**

As a mitochondrial toxin, rotenone can cause oxidative stress and activate apoptotic pathways in rats after prolonged exposure. The elevated glutamate and aspartate levels in rats are linked to the excitotoxicity caused by rotenone. Rats treated post-rotenone treatment showed lower glutamate and aspartate levels than control and individual neuroprotectant-administered rats, indicating that EWSR and other neuroprotectants have anti-excitotoxicity or anti-inflammatory properties. It can also be assumed that these combinations of neuroprotectants could be an alternative source for reducing excitotoxicity or neuroinflammation. The findings of this study are consistent with previous research, and we were able to lessen the rotenone-induced excitotoxicity by administering EWSR alone or in conjunction with well-known neuroprotectants. This was done by lowering the levels of glutamate and aspartate **Fig. 8** and **9** [38, 40, 46].

CONCLUSION: *Withania somnifera* (WS)is an ayurvedic plant whose beneficial properties are well established over the last few decades of considerable in vitro and in vivo research, and it is believed that WS can contribute significantly in the treatment of Parkinson's disease by lowering oxidative stress, enhancing cholinergic function, and enhancing mitochondrial respiratory mechanisms, it has been shown that a standardized extract of WS can significantly lessen the locomotor defects and lethality of rotenone-induced Parkinsonism in *Drosophila melanogaster*. Through its anti-inflammatory and anti-oxidant qualities and resolving mitochondrial dysfunctions in the striatum and cerebellum regions, WS can also significantly reduce rotenone-induced parkinsonism in the brain by conditioning the dopamine levels and neurotransmitter activity in the striatum.

Rotenone exposure in rats over an extended period of time led to behavioural deficiencies (memory, learning, cognition, anxiety, emotional activity, and motor control), as well as differences in neurotransmitters. The levels of the neurotransmitters glutamate and aspartate increased with chronic fluoride exposure in the postnatal rats, which agrees with earlier studies on male and female rodents treated with Rotenone.

The brain bioamines EPN, NE, and DA levels significantly decreased during chronic rotenone exposure and were similar to Ach. Following rotenone treatment, the administration of EWSR and other neuroprotectants considerably reduced the toxic effects of rotenone on the rat brain. It assisted in correcting the motor, behavioural, and memory features of the rats by restoring the neurotransmitter levels to normal. Our present observations indicate the beneficial and neuroprotective efficacy of EWSR individually and in combination with known anti-inflammatory agents. The present findings are in coherence with the published literature, but further research is required to confirm the underlying neuroprotective, anti-neuroinflammatory mechanisms of EWSR's effective effects before taking into the clinics.

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